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Mechanism of Silver Sulfadiazine Action on Burn Wound Infections

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The role of silver and sulfadiazine in the mechanism of action of silver sulfadiazine on burn wound infections was investigated. Silver, but not sulfadiazine, was bound by bacteria. Sulfadiazine did not act as an antibacterial agent in low concentrations, but exhibited specific synergism in combination with subinhibitory levels of silver sulfadiazine. The efficacy of silver sulfadiazine is thought to result from its slow and steady reactions with serum and other sodium chloride-containing body fluids, which permits the slow and sustained delivery of silver ions into the wound environs. In this circumstance, a relatively minute amount of sulfadiazine appears active.

Although silver sulfadiazine (AgSD) has received wide-spread acceptance as a topical agent to control bacterial infection, especially in burn wounds (2, 6-9, 13-15, 19, 20), and is now approved by the Food and Drug Administration, its mechanism of action is uncertain.

This compound was prepared to combine the oligodynamic action of silver with the antibacterial effect of sulfadiazine (7). Subsequent studies (9) showed that the sulfonamide antagonist para-aminobenzoic acid (PAB) did not nullify silver sulfadiazine inhibition, and that the silver moiety combined in vitro with both DNA and bacteria. The sedimentation coefficient of DNA isolated from AgSD-inhibited bacteria was found to be higher than that of normal DNA (22). Subsequently, Carr and Rosenkranz (4) reported that AgSD became bound to the cell membrane and suggested that the resulting membrane damage caused the bactericidal action observed. The failure of PAB to block growth inhibition and the relatively low concentration of AgSD required made the role of the sulfadiazine uncertain. This was studied by experiments with ^{110}AgSD and Ag^{35}SD which showed that the AgSD dissociated, that Ag alone became bound to various components within the cell and that the inhibition of bacterial growth was related quantitatively to the binding of Ag to microbial DNA (16).

This communication indicates the probable roles of silver and sulfadiazine and compares the reactions of AgSD, less effective silver sulfonamides and other silver salts with sodium chloride, DNA, human serum, and broth. The synergism of AgSD and SD is also shown. The observations may account for the unique efficacy of topical AgSD in preventing and treating infections and may help elucidate its mechanism of action in burns and infected wounds.

MATERIALS AND METHODS

Silver sulfadiazine was prepared as described previously (7).

Radioactive silver salts. The radioactive silver salts were prepared by reacting the sodium salts with ^{110}AgNO$_3$ obtained from the International Chemical and Nuclear Corp. The silver salts thus prepared had a specific activity of about 1.0 mCi/mmol.

Radioactive sulfadiazine. This was kindly supplied by Fred Williams of the Radiological Services Group of Iowa State University as Na^{35}SD. The Ag^{35}SD prepared from it had a specific activity of 1.0 mCi/mmol; the Na^{35}SD was also used as such in some of the experiments after dilution to the same specific activity.

Bacterial strains. The *Pseudomonas aeruginosa* strain used in most of our experiments was obtained originally from Donald P. Dressler with the designation WHTG no. 2. This is highly virulent to both rats and mice and was used in our previous studies (9, 11, 16). The *Staphylococcus aureus* strains were isolates obtained from hospital patients.

DNA solution. Calf thymus DNA (Worthington-Biochemicals) dissolved in 0.005 M NaNO$_3$ and dialyzed against 0.005 M NaNO$_3$, was used in all the experiments.

Preparation of Sephadex column. To 100 ml of water, 5 g of Sephadex G-100 with a particle size of 40 to 120 μm (Pharmacia Fine Chemicals) was added gradually and stirred to obtain a uniform suspension. The suspension was then heated in a boiling water bath for 8 h and stored at 10 C. Columns of 20-cm length and 0.5-cm diameter were prepared with this Sephadex and washed several times with water. (Note: When a column of 20-cm length and 0.5-cm diameter was used, up to 0.2 μmol of free silver was retained on the column while the bound silver [Ag-DNA complex] passed through the column. Accordingly, at no time did the samples loaded on a column contain more than 0.2 μmol of silver.)

Separation of Ag-DNA complex on Sephadex. The DNA solution was mixed with the desired amount of AgSD or $AgNO_3$ and incubated at 37 C for 20 h. The reaction mixtures were then centrifuged at 8,000 rpm for 10 min, and portions of the clear supernatant were passed through the column. Elution was done with water, and 15 tubes, each containing 2 ml of eluate, were collected. All tubes were read at λ260 nm in a Beckman DU spectrophotometer, analyzed for radioactivity, and the separation pattern was determined. Inasmuch as the binding of silver to DNA shifted and increased the ultraviolet (UV) absorption of DNA (12), accurate measurement of DNA for calculating the Ag/DNA ratio of the complex was done by using the specific diphenylamine reaction (3).

Radioactivity measurements. The radioactivity of ^{110}Ag was measured in a well-type scintillation detector with a 1-inch diameter (thallium activated) sodium iodide crystal connected to a Nuclear-Chicago 720 series counter. The radioactivity of ^{35}S was measured by using an Aquasol scintillator in a Nuclear-Chicago 720 series liquid scintillation counter.

RESULTS

Binding of sulfadiazine from Na^{35}SD by Pseudomonas and Staphylococcus. During inhibition with AgSD, no bacterial binding of SD occurred. Inhibition by NaSD, however, requires considerably higher concentrations of drug and utilizes a different mode of action, namely, PAB antagonism, and binding of SD might occur. The inhibitory concentration of sulfadiazine (SD) for *Pseudomonas* and *Staphylococcus* and the binding of ^{35}SD by these organisms is shown in Table 1. For both organisms 5 μmol/ml was partially inhibitory and 15 μmol/ml was completely inhibitory. It is seen that even at this high inhibitory concentration the binding of ^{35}SD was negligible for both *Pseudomonas* and *Staphylococcus*.

Although the binding at the inhibitory concentration was less than 1% of the amount of drug present, this represents a 100-fold increase over the minute uptake when inhibition did not occur. In contrast, during inhibition with AgSD (0.1 μmol/ml), no SD uptake occurred although as much as 20% of the ^{110}Ag was taken up (see Table 6, ref. 8).

Inasmuch as the solubility of SD in water is only 0.5 μmol/ml, these considerably higher concentrations needed for bacteriostasis were attained by virtue of the fact that the pKa of sulfadiazine is 6.1, and at pH 7.0, most of the drug is in the form of NaSD (10). Significantly, the available H^+ has been exchanged for Ag in AgSD and this solubility transformation cannot occur (except with decomposition) at extremely low or high pH.

Studies indicating the dissociation of AgSD prior to binding with DNA. The dissociation of AgSD has been further confirmed by studying the binding of Ag and SD to DNA by three different methods.

(i) Equilibrium dialysis. The binding to DNA of radioactive sulfadiazine from Ag^{35}SD and Na^{35}SD and of radioactive silver from ^{110}AgSD was studied by using an equilibrium dialysis technique. The results depicted in Table 2 show that no SD became bound to DNA either from Ag^{35}SD or Na^{35}SD; in sharp contrast, almost 25% of ^{110}Ag from ^{110}AgSD became bound to the DNA.

(ii) Separation in Sephadex column. The elution pattern of Ag^{35}SD-DNA complex is shown in Fig. 1. The DNA was eluted in tubes 2 to 5 and carried no radioactivity from ^{35}SD; the SD was eluted in tubes 6 to 11, and these contained all the radioactivity.

The elution patterns of ^{110}Ag-DNA complexes from ^{110}AgSD and $^{110}AgNO_3$ are shown in

TABLE 1. *Binding of sulfadiazine by Staphylococcus and P. aeruginosa treated with sodium sulfadiazine*[a]

Concn of drug (μmol/ml)	*Pseudomonas*		*Staphylococcus*	
	Growth (OD)	% Uptake	Growth (OD)	% Uptake
0	1.0		0.9	
1.0	1.0	0.008	0.9	0.005
2.0	0.95	0.010	0.9	0.005
5.0	0.40	0.075	0.58	0.05
10.0	0.30	0.10	0.40	0.08
15.0	0.15	0.80	0.15	0.40

[a] A 50-ml amount of early log phase cells (0.15 OD) was distributed in 250-ml Erlenmeyer flasks containing the desired amount of Na^{35}SD (radioactivity in each flask corresponds to 500,000 counts/min) and incubated at 37 C on a shaker. After 3 h, the flasks were removed and the cells were collected by centrifugation, washed twice with sterile broth, and then with water. Then the radioactivity in the washed cell mass was determined.

Fig. 2. These solutions or suspensions were mixed with DNA in the ratio, Ag/DNA-P = 1. The DNA eluate in tubes 2 to 5 carried the ^{110}Ag radioactivity, with no radioactivity in the SD region (tubes 6 to 10). The DNA reacted with soluble $^{110}AgNO_3$ acquired more radioactivity than DNA reacted with slightly soluble ^{110}AgSD.

(iii) UV absorption of DNA. If AgSD binds as an intact molecule to DNA, the UV absorption of AgSD-DNA complex is expected to be greater than the $AgNO_3$-DNA complex of the same Ag/DNA ratio. This was studied by comparing the UV absorption of Ag-DNA complex from $AgNO_3$ and AgSD. DNA was mixed with AgSD or $AgNO_3$ at different Ag/DNA-P ratios and incubated for 20 h. After centrifuging, the UV absorption of the supernatant was read against 0.005 M $NaNO_3$. Sulfadiazine absorbs strongly at λ260 nm, and therefore each supernatant was analyzed for free, unreacted sulfadiazine. The supernatants were passed through Sephadex columns, and the eluates containing the free sulfadiazine were analyzed by the Bratton-Marshall colorimetric method (1). The OD_{260} measured for the DNA-AgSD mixtures was then corrected by subtracting the OD_{260} calculated for free sulfadiazine based on the colorimetric analysis. When $AgNO_3$ was used, this correction was unnecessary.

As shown in Table 3, the UV absorption of the Ag-DNA complex formed from AgSD was similar to that formed from $AgNO_3$ at the lower Ag/DNA-P ratios. The UV absorption of the DNA was not increased by either silver compound until the Ag/DNA-P ratio in the reaction mixture was about 0.25.

At this ratio the Ag-DNA complex formed from $AgNO_3$ showed an increase in absorbance, and higher ratios showed proportionately greater increases. (This agrees with the report of Jensen and Davidson [12] which indicated increased absorbance at a ratio of 0.26 or above.) However, the Ag-DNA complex formed from AgSD showed no such increase until the Ag/DNA-P ratio reached 1.0. This difference in absorbance of the two silver salt complexes was found to be due to differences in the Ag/DNA ratio of the complex itself, determined by Sephadex column separation as explained in Materials and Methods. The complexes resulting

TABLE 2. *Binding of silver and sulfadiazine to DNA by equilibrium dialysis*[a]

Compounds	Radioactivity associated with DNA (% of total radioactivity)
$Na^{35}SD$	0
$Ag^{35}SD$	0
^{110}AgSD	25

[a] Dialyzing bag contained 3 ml of DNA at a concentration of 300 μg/ml. Dialysis was carried out for 20 h against 200 ml of 0.5 μmol of the above compounds in ammoniacal solution per ml, pH 8.0. (Total radioactivity corresponds to 500,000 counts/min.) The radioactivity of the solutions inside and outside the bag was measured.

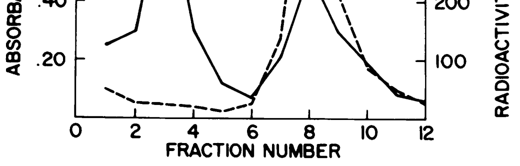

FIG. 1. *DNA solution (35 μg/ml) was mixed with $Ag^{35}SD$ (silver/DNA-P = 1.0) and incubated for 20 h. After centrifugation a portion of the clear supernatant was loaded on a Sephadex column. Symbols: OD, ——; -----, radioactivity.*

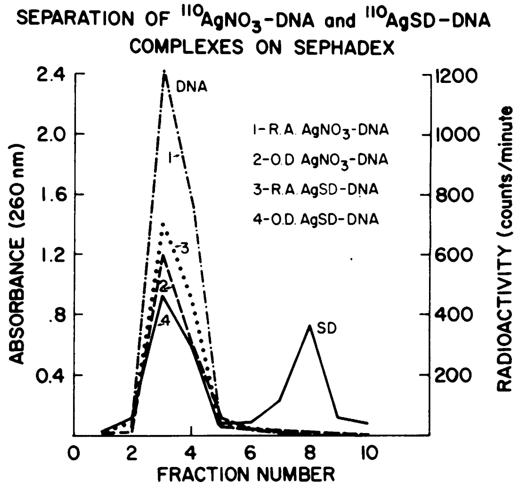

FIG. 2. *DNA solutions (35 μg/ml) were mixed with ^{110}AgSD or $^{110}AgNO_3$ (silver/DNA-P = 1.0) and incubated for 20 h. After centrifugation, a portion of the clear supernatants was loaded on a Sephadex column. R.A., Radioactivity.*

TABLE 3. *Effect of silver binding on ultraviolet absorption of DNA*

Ag/DNA-P in reaction mixture	AgSD				AgNO3		
	Binding ratio[a] (μmol of Ag/100 mg of DNA)	OD_{260}	OD_{260} after correaction for SD absorption[b]	Increase in OD caused by Ag	Binding ratio[a] (μmol of Ag/100 mg of DNA)	OD_{260}	Increase in OD caused by Ag
0	0	0.68	0.68	0	0	0.68	0
0.125	22	0.88	0.66	0	38	0.67	0
0.25	33	1.0	0.67	0	64	0.71	0.03
0.5	48	1.15	0.68	0	96	0.79	0.11
1.0	60	1.23	0.70	0.02	120	0.81	0.13

[a] The binding ratios were estimated from the ^{110}Ag taken up by the DNA.
[b] Free SD determined in the supernatant as described in Results.

from $AgNO_3$ attained consistently higher Ag/DNA ratios than those from AgSD, doubtless due to the greater solubility of $AgNO_3$.

Thus, all three experimental methods indicated that AgSD dissociates upon combining with DNA, and that the SD moiety does not combine.

Reactions of silver salts with sodium chloride, nutrient broth, human serum, DNA, and bacteria. These reactions were studied to visualize the clinical experience in complex wound exudates.

Silver sulfadiazine and other silver salts were incubated with 0.01 M NaCl, nutrient broth, human serum, DNA, and bacterial suspensions, and at intervals the cations and anions released into the supernatant were measured. The percentage of silver salts unreacted after incubation with serum for various time intervals is shown in Fig. 3. The reaction rates of these silver salts in the presence of the other test substances were similar to the patterns depicted in serum and hence are not shown here. The silver salts of sulfanilamide, uracil, and nitrate reacted almost completely within 15 min. Ag sulfacytosine, Ag sulfathiazole, and Ag sulfamerazine reacted very slowly, but silver sulfadiazine reacted at a moderate rate which continued so that 20% still remained unreacted after 40 h.

Binding of silver by growing cultures of Pseudomonas containing the supernatant and sediment fractions of serum reacted with silver salts. To simulate further the conditions existing in wound exudates, silver sulfadiazine and other silver salts were incubated with normal human serum and were subsequently exposed to bacteria. The serum-silver salt mixtures were centrifuged after incubation, and the supernatants (containing drug in solution) and sediments containing undissolved drug were

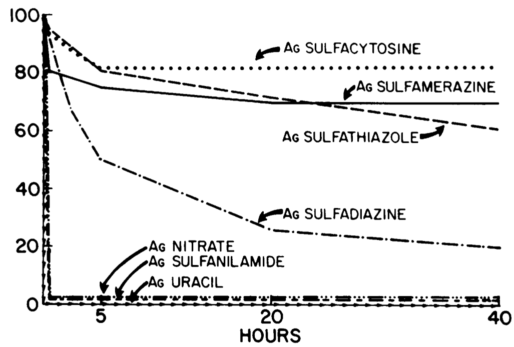

FIG. 3. *Human serum was incubated with 10 μmol of each silver salt. At the intervals designated, portions were removed and centrifuged, and the clear supernatants were analyzed. Silver was determined by ^{110}Ag counts and sulfonamides by the colorimetric procedure (1). The amount of compound used was taken on 100%.*

collected. Portions (corresponding to 0.1 μmol of silver based on actual counts and specific activity of ^{110}Ag used) were taken from both these supernatants and sediments to determine the binding of silver by bacteria. For this purpose each fraction received 5 ml of early log phase cultures (0.4 to 0.5 OD) and all were incubated for 3 h. The tubes containing the silver salt residues were then given a preliminary centrifugation at 2,000 rpm for 5 min, after which the residues of unreacted silver salts were discarded, and the supernatants containing the bacteria were pipetted off. These supernatants were again centrifuged, at 8,000 rpm for 10 min, and the silver uptake was measured on the sedimented bacterial cell mass by counting the associated radioactivity.

The tubes incubated with the silver salt supernatants were handled in the same way, except that the preliminary centrifugation was unnecessary.

The bacterial binding of silver from the supernatants and sediments of the various silver salts reacted with serum are shown in Table 4. The bacteria bound similar amounts of silver from the supernatant fractions (15 to 18%) of all the silver salts. The bacterial binding from the sediments was higher; the maxima (74 and 83%, respectively) were attained with silver nitrate and silver sulfadiazine.

Synergism of silver sulfadiazine and sodium sulfadiazine against Pseudomonas aeruginosa. The combinations of AgSD and SD used are shown in Table 5. When the inhibitory level of SD was reduced from 0.6 to 0.4 μm/ml, inhibition was restored by adding 0.002 μm of AgSD per ml. When the level of AgSD was reduced from 0.01 to 0.002 μm/ml, inhibition was regained by adding 0.4 μm of SD per ml. When the concentration of AgSD was reduced from 0.01 to 0.004 μmol/ml, inhibition was regained by adding 0.4 μm of SD per ml, a sub-inhibitory concentration. These data suggest that 1 μm of AgSD per ml can be substituted for by 50 μm of SD per ml or vice versa. Thus, a sub-inhibitory amount of NaSD (0.4) becomes inhibitory upon addition of a non-inhibitory amount (0.002) of AgSD. Likewise, a sub-inhibitory level of AgSD (0.006) becomes inhibitory upon addition of approximately one-sixth the inhibitory level of NaSD. In contrast, a sub-inhibitory (one-half inhibitory level) amount of sulfathiazole could not be substituted; this suggests that the synergism observed is specific for sulfadiazine.

TABLE 4. *Binding of silver from residual and supernatant fractions of serum reacted with silver salts by growing culture of Pseudomonas*

Silver salts	Percentage of silver bound to the bacteria[a]	
	Residue	Supernatant
Silver sulfanilamide	52	15.0
Silver sulfacytosine	40	14.5
Silver sulfamerazine	58	14.8
Silver sulfathiazole	54	18.5
Silver nitrate[b]	74	16.8
Silver chloride[b]	76	15.0
Silver sulfadiazine	83	15.5

[a] The percentage of silver bound to the bacteria was calculated using the initial ^{110}Ag counts in each residue and each supernatant as 100%.

[b] Handled in dark to minimize rapid decomposition and darkening in light.

TABLE 5. *Synergism of silver sulfadiazine and sodium sulfadiazine against P. aeruginosa*

Compounds (μmol/ml)	Effect on growth[a]	Compounds (μmol/ml)		Effect on growth
		AgSD	NaSD	
AgSD				
0.004	+	0.001	0.5	+
0.006	+	0.001	0.6	−
0.008	+			
0.01	−	0.002	0.1	+
		0.002	0.2	+
NaSD		0.002	0.4	−
		0.002	0.6	−
0.2	+	0.002	1.0	−
0.4	+			
0.5	+	0.004	0.1	+
0.6	−	0.004	0.2	+
1.0	−	0.004	0.4	−
		0.004	0.6	−
NaST		0.004	1.0	−
0.025	+	0.006	0.1	−
0.05	+			
0.1	−	0.008	0.025	+
0.2	−	0.008	0.05	+
		0.008	0.1	−
		AgSD	NaST	
		0.002	0.025	+
		0.002	0.05	+
		0.002	0.1	−
		0.002	0.2	−
		0.004	0.05	+
		0.006	0.05	+

[a] +, Growth; −, no growth; NaST, sodium sulfathiazole. The inoculum was 0.2 ml of 1/2,500 dilution of an overnight Trypticase soy broth culture in 5 ml of broth containing the drugs. The cultures were incubated for 20 h.

DISCUSSION

The dissociation of AgSD during inhibition of bacterial growth has been described (16) but to determine the role of each constituent, confirmation by other experimental methods is essential. It is now clear that no significant amount of SD entered the bacterial cells (either *Pseudomonas* or *Staphylococcus*) even during inhibition by the much higher concentrations of NaSD. It is thus unlikely that SD entry in the cell occurs at the lower concentrations used for AgSD inhibition.

It was further found by three different methods that silver-DNA complexes formed from AgSD and DNA in vitro contained no SD.

TABLE 6. *Effectiveness of various compounds as tested against Pseudomonas infection in mice (30% burn)*

Compound	No. of mice	Mortality
Untreated[a]	132	88.2
Ag penicillin	40	100
Ag cephalothin (Keflin)	30	100
Ag colistin	20	100
Ag acetate	40	100
Ag aminobenzoate	20	100
Ag nitrobenzoate	20	100
Ag salicylate	30	70
Ag ethylene diamine tetra-acetic acid (EDTA)	20	100
Ag fluorouracil	40	73
Ag sulfathiazole	20	40
Ag sulfamethizole	20	65
Ag sulfamerazine	20	90
Ag sulfasomidine	20	60
Ag mafenide	20	100
Mafenide HCl or acetate (Sulfamylon)	70	74.5
Silver sulfadiazine[a]	144	25.6
Silver nitrate (0.5%)	100	67.5
Silver sulfacytosine	20	90
Silver sulfanilamide	20	77
Silver uracil	20	90
Sulfadiazine sodium (1%)	50	80
Sodium chloride (0.9% solution)	20	90
Ointment base	20	65

[a] These have been compared in numerous subsequent experiments (11) with larger numbers of mice and rats.

Thus, although SD does not appear to function chemically in the bactericidal action of AgSD, in infected burns in animals this compound proved superior to the many other silver compounds prepared and tested. All the silver compounds listed in Table 6 showed strong antibacterial action in vitro, but proved ineffectual in vivo.

It seemed plausible that this efficacy of the SD combination with Ag might be due to a difference in dissociation and/or reaction rate under physiologic conditions. To explore this possibility, the reactions of various silver salts with sodium chloride, nutrient broth, DNA, bacteria, and human serum were studied. Most of the compounds either dissociated completely with rapid removal of all silver, or else dissociated only slightly without appreciable release of silver over a period of time. AgSD, on the contrary, showed a moderate initial dissociation, with continual release of silver over the time observed. The ionization constants of the sulfonamides (10), and possibly of the other compounds also, would appear to contribute importantly to the differences in the antibacterial activity of their silver salts.

To estimate the obtainable silver ions on the wound surface when these different silver salts were used, the bacterial binding of silver from the supernatant and sediments of human serum reacted with these compounds was measured. Considerable amounts of AgSC, AgSM, and AgST remained unreacted in the sediment; the uptake from these compounds was much less, presumably because of their slow dissociation rates, hence the non-availability of silver ions. The maximum silver binding was obtained from $AgNO_3$ and AgSD. With $AgNO_3$, the sediment may constitute precipitated silver proteinate and silver chloride which are capable of releasing free silver ions and producing the characteristic black staining.

The similarity of binding from the supernatants of all these compounds suggests that any large amounts of silver released were precipitated as silver proteinate and AgCl and remained sedimented. In the case of AgSD, the sediment consisted mostly of unreacted silver sulfadiazine. Thus, silver sulfadiazine functioned as a reservoir of obtainable silver in the wound. This slow liberation of silver ions does not cause the rapid and extensive depletion of chloride ion experienced with continuous silver nitrate solution soaks, and hence systemic electrolyte withdrawal is avoided (2, 8). Since only a small part of the silver in AgSD reacted with the chloride, protein and other constituents of body fluids, enough was available to be acquired by microorganisms, so that many were killed and their growth inhibited. The correspondingly low level of sulfadiazine proved innocuous and practically no silver was absorbed. (2, 9).

However, it is not proposed that this difference of availability of Ag from the precipitate represents the complete picture of what occurs in the wound; but it may represent the trend of the reactions taking place.

The unique role of SD is further illustrated by the synergism experiments with AgSD, which sulfathiazole could not mimic.

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